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Immunology in the clinic review series; focus on cancer: double trouble for tumours: bi-functional and redirected T cells as effective cancer immunotherapies

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Summary

Cancer is one of the most important pathological conditions facing mankind in the 21st century, and is likely to become the most important cause of death as improvements continue in health, diet and life expectancy. The immune response is responsible for controlling nascent cancer through immunosurveillance. If tumours escape this control, they can develop into clinical cancer. Although surgery and chemo- or radiotherapy have improved survival rates significantly, there is a drive to reharness immune responses to treat disease. As T cells are one of the key immune cells in controlling cancer, research is under way to enhance their function and improve tumour targeting. This can be achieved by transduction with tumour-specific T cell receptor (TCR) or chimaeric antigen receptors (CAR) to generate redirected T cells. Virus-specific cells can also be transduced with TCR or CAR to create bi-functional T cells with specificity for both virus and tumour. In this review we outline the development and optimization of redirected and bi-functional T cells, and outline the results from current clinical trials using these cells. From this we discuss the challenges involved in generating effective anti-tumour responses while avoiding concomitant damage to normal tissues and organs.

Keywords: bi-functional, cancer, CAR, redirected, T cell therapy

Introduction

Cancer is one of the most important pathological conditions facing mankind in the 21st century. It is second only to heart disease as the biggest cause of mortality in the developed world, and is likely to become the most important cause of death as improvements continue in health, diet and life expectancy [1]. The immune response is responsible for controlling nascent cancer through immunosurveillance [2]. In the control of cancer, both the innate and adaptive arms of the immune system act together to co-ordinate the eradication of the developing tumour. If these early microtumours are not cleared completely by this concerted immune attack then a period of uneasy equilibrium develops, which can exist for significant periods of time [3]. Eventually the inherent genetic instability in tumour cells leads to the development of immune evasion mechanisms – loss of tumour-associated antigens (TAAs) or down-regulated MHC antigen expression, T cell inactivation through reduced T cell receptor (TCR) signalling or interleukin (IL)-10 and transforming growth factor (TGF)-β-mediated suppression [2,4] *inter alia*.

These mechanisms induce a state of immune tolerance and inactivate tumour-specific T cells, rendering the immune system incapable of recognizing TAAs and mounting an effective immune response against them. This low immunogenicity and subsequent induction of immune tolerance remains the most difficult obstacle to overcome when establishing an effective cancer immunotherapy.

Adoptive cellular therapy (ACT) offers the hope of manipulating the immune system to re-ignite the tolerized immune response of the patient against the TAAs. ACT ideally involves the administration of autologous or allogeneic lymphocytes which have been manipulated and expanded *ex vivo* to exhibit high specificity for TAAs [5]. ACT is effective against highly metastatic and vascularized cancers which are otherwise very difficult to treat [5]. The

first major advance in ACT came in 1988, with the treatment of patients with metastatic melanoma with autologous tumour-infiltrating lymphocytes (TIL) following lymphodepletion, and development has continued to produce effective therapy [5–7]. While successful in the treatment of metastatic melanoma this method is not easily transferable to other cancers, where TILs cannot easily be identified and expanded [8]. By their nature, tumour-specific T cells are often difficult to render into an effective state, and this has pushed research to look for a genetic engineering solution to this problem. In this review we will look at the transduction of new specificities into T cells, the different methods that can be employed and the evidence for the effectiveness of this treatment.

Adding antigen-specificity – redirected-specificity T cells

The tolerizing of the immune system to tumour antigen is a multi-faceted problem. Tumour antigens can be cryptic, poorly presented, of low affinity, lost or rapidly shed [2,9]. Genetically engineering antigen-specificity into T cells is a powerful way to combat tolerance and can be achieved in a number of ways: through TAA-specific TCR gene transfer; introduction of a chimaeric antigen receptor (CAR) redirecting the T cell to recognize the tumour; and transduction of a TAA-specific TCR or CAR into T cells which are already directed against another specific antigen (bi-functional T cells) [10].

TCR gene transfer

Where major histocompatibility complex (MHC)-presented antigens have been identified, the transfer of T cell receptor (TCR)-αβ chains from a T cell with high affinity for a defined TAA (either from rare human clones or from mice following immunization) into a new T cell using an integrating vector creates a redirected T cell which has both an endogenous (natural) TCR and an exogenous (introduced) TAA specific TCR [5,11]. TCR-αβ chains contain variable (V) and constant (C) regions, which are key in the binding of peptide-bound MHC (pMHC) on antigen-presenting cells (APCs) [11,12]. TCR-αβ transduced cells are generally MHC-restricted and thus target an identical human leucocyte antigen (HLA) phenotype TCR, although transduced T cells have been generated which are non-MHC-restricted, or allogeneic rather than autologous [13,14].

TCR gene transfer has been used in clinical trials for various tumours possessing distinct TAAs. In 2006, Morgan *et al*. reported full tumour regression in two of 14 patients in the first Phase 1 clinical trial using the DMF4 TCR specific for the melanoma TAA melanin A (MART-1) [8]. In total, 31 patients were eventually treated with objective regression of tumour observed in four (13%) of the patients [15]. Disappointingly, this level of clinical response was significantly lower than the 50–70% response rate observed with TIL therapy performed by the same group [7]. More recently, five of 11 melanoma and four of six patients with metastatic synovial sarcoma demonstrated complete tumour regression after treatment with T cells engrafted with a NY-ESO-1-specific TCR [16].

In an effort to improve the efficacy of these redirected T cells, a combination of improved vector design and TCR of higher affinity was tested in a subsequent melanoma trial. The improved DMF5 MART-1 TCR and a mouse gp100 TCR each conferred an approximately 100-fold increase in sensitivity to peptide concentration and displayed improved effector function *in vitro* compared with the T cells bearing the DMF4 TCR. While patients treated with these improved T cells demonstrated an improved clinical objective response (DMF5: 30% response in 20 patients treated; gp100: 19% response in 16 patients treated), this level of response still failed to reach the levels achieved with TIL therapy [15].

Moreover, a significant degree of on-target toxicities were observed with both TCRs in the form of skin depigmentation and rash, uveitis, mild hearing loss and ear-related dizziness [15]. Depigmentation has been seen commonly in melanoma-targeted therapies as a result of targeting of melanocytes present in the skin. Melanocytic cells are present in the eye and ear, and it is these cells that may have been targeted by the transduced T cells. However, no toxicity of the ear or eye was observed in patients treated with the DMF4 TCR despite the peptide target for both DMF4 and DMF5 TCRs being the same epitope. Recent evidence has shown that this altered pathological response may be due to a distinct difference in the way that the two TCRs bind to the same peptide [17]. The issue of on-target toxicity was also evident in a small trial employing a carcino-embryonic antigen (CEA)-specific TCR. One patient of three treated showed objective regression of lung and liver sites of colon cancer metastasis. However, all three patients suffered a severe transient colitis [18].

Taken together, more than 80 patients have been treated with T cells transduced to express TCRs, demonstrating the proof of principle that the clinical delivery of this therapy is possible. However, this work has been performed solely at one site: the Surgery Branch, NCI, Washington. TCR-based clinical trials are at the advanced planning stage in several other centres around the world and the results of these will be important to establish further the clinical efficacy of the treatment and also to establish the feasibility to provide this treatment globally.

Several issues still need to be overcome in order to establish TCR gene transfer as an effective cancer immunotherapy. Some of these issues include the choice of T cells used for transfer, the transfection vector used and the target antigen.

Identifying the most effective method of introducing TCR-αβ chains into a vector (the transgene cassette) needs to be established and several approaches have been adopted,

Table 1. Issues with TCR gene transfer.

Problems	Solution	Mechanism of action	Disadvantages
Mispairing			
Mispairing of introduced TCR with endogenous TCR-αβ chains may form non-sense or potentially autoreactive receptors [19,22]	Add cysteine residues in the C region of the introduced TCR-αβ chains [19]	Promotes preferential pairing of introduced TCR-αβ chains [13]. Forms a disulphide bridge resulting in increased quantity of transduced TCR-αβ pairs and percentage of matched chains [11,19,53]	Cysteine-TCRs show stronger binding than normal, but no correlation with increased function [71]
Low CD3 affinity			
Introduced TCRs need very high affinity for pMHC due to competition with endogenous TCR [12]	Murinization [11,12]	Replacing human TCR C domains with murine domains which bind to CD3 with higher affinity without affecting transduced TCR specificity [11,12,72] Murine and human TCR persist for equivalent time [15]	Murine sequence potentially immunogenic unless TCR is murinized only at essential C region residues. Lys18 and Ala22 are crucial for superior TCR function [73]
Low expression of introduced TCR	Optimize codons of TCR genes [11,12]	Exclusion of cryptic splice sites and mRNA instability motifs from the transduced TCR increases efficacy and expression [12]	Process alters gene sequence and gives possibility of immunogenicity [19]
Low avidity of introduced TCR	Reduce N-glycosylation of TCR chains [74]	Removal of certain motifs in C regions results in increased avidity, cytokine secretion and cytolytic activity. Increased binding with pMHC [74]	Regulation of N-glycosylation is thought to vary according to the differentiation state of the T cell [74]

pMHC, peptide-bound major histocompatibility complex; TCR, T cell receptor.

such as single or dual TCR vectors, with and without individual promoters (reviewed by Uckert and Schumacher [19]).

However, introduced TCRs can often be ineffective *in vivo* due to factors such as low expression [12]. TCR-αβ chains pair in the most favourable combination, and hence TCR cell surface expression is controlled by the presence of both endogenous and introduced TCRs, so efficiency is irrespective of whether the TCR is endogenous or introduced [20]. Many of the problems with TCR gene transfer have promising solutions, which are outlined in Table 1. A study by Hart *et al.* [21] demonstrated that the expression and efficacy of 'weak' TCRs can be improved using a combination of murinization, linking with amino acid sequence and codon optimization, highlighting the important advancements made in this field. Mispairing of transferred TCR with endogenous TCR has been shown to lead to potentially lethal autoreactivity [22]. Although advances in research in this area (addressed in Table 1) have produced methods to optimize TCR transfer techniques, other approaches have also been adopted by researchers interested in generating more powerful T cell responses to cancer.

The issue of on-target toxicity remains a major problem. The potential to eradicate sizeable, established tumours will be dependent upon ensuring that the adoptively transferred T cells possess optimal potency for antigen. However, where this antigen is not tumour-specific and is expressed on healthy tissue, albeit at lower absolute levels than on the tumour, then there are clearly issues with on-target toxicity. Understanding the balance between optimal redirected T cell function (TCR affinity, level of expression, vector optimization) against toxicity is clearly important for the future widespread application of this approach.

Chimeric antigen receptors (CAR)

CAR utilize a different approach to generate a redirected T cell, where the introduced TCR gene is generally replaced by a multi-component construct, generated normally (but not exclusively) using the antigen-binding domains from the variable regions of a TAA-specific monoclonal antibody (ScFv) linked to T cell signalling domains, as shown in Fig. 1. CAR have an advantage over TCR gene transfer in that they are not MHC-restricted and do not need to be HLA-matched to the patient, producing an anti-tumour response even when tumour cell MHC molecules have been down-regulated [4].

The scFv is linked generally to the transmembrane domain via an extracellular linker domain such as the immunoglobulin (Ig)G Fc hinge region or extracellular CD8 section. These domains may enhance flexibility and dimerization of the receptor [23–25]. There is some evidence that these domains can engage other cells such as macrophages or natural killer (NK) cells by binding via the Fc receptor leading to a proinflammatory response irrespective of CAR binding, although this can be minimized by modifying the

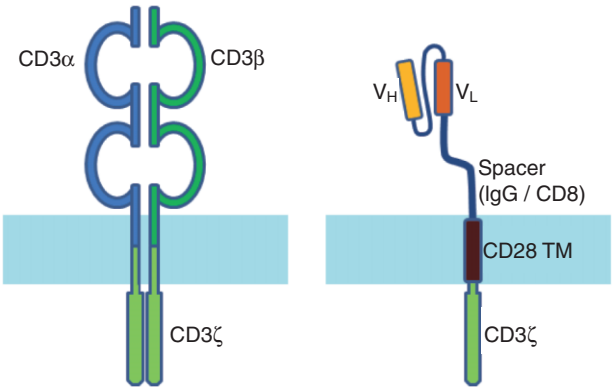

Fig. 1. Representation of a conventional T cell receptor in comparison with a basic chimeric antigen receptor (CAR). The CAR is composed of linked V chains from a tumour-associated antigen (TAA)-specific monoclonal antibody, bound to a spacer arm from the hinge region of immunoglobulin (Ig)G or from the CD8 receptor. This then attaches to a CD28 transmembrane domain (TM) [23–25]. The cytoplasmic domain is composed of the CD3-ζ signalling element, although other elements may also be included, such as CD28 and 4-1BB motifs which give improved activation and survival [23–25].

constant domain of the Fc region [25,26]. However, there is also evidence to suggest that the position of the targeted epitope relative to the target cell membrane is important with a flexible extracellular spacer region on the CAR required for targeting epitopes buried close to the membrane. Often, no spacer is required for CAR targeting epitopes at the distal regions of cell surface proteins [27]. Additionally, a flexible elongated hinge region may be necessary to overcome the steric inhibition imposed by large tumour antigens [28]. Consequently, the optimal arrangement of scFv and extracellular spacer still requires a degree of empirical testing.

First-generation CAR incorporated a single signalling domain which most commonly used the intracytoplasmic domain of CD3ζ. T cells armed with this CAR demonstrated redirected effector function against target cells expressing the antigen of choice. While T cells armed with these basic receptors can function effectively *in vivo* to prevent the growth of tumours in models, they fail to persist in the long term [29].

The modular system of engineering CAR lends itself to incorporating multiple signalling domains. Improvements in signalling, proliferation and survival were observed when the cytoplasmic CD3ζ element was coupled with a CD28 signalling domain [30]. These so-called second-generation CAR demonstrated the benefits of combining signalling elements to harness both activation and co-stimulation mechanisms. The CD28 domain also confers resistance to regulatory cells often found in the cancer microenvironment [31].

CAR optimal co-stimulation requires sequential ligation of CD28 followed by another signal such as OX40 (CD134 co-stimulatory receptor on APCs) [24]. CAR using OX40, inducible co-stimulator (ICOS) or more recently triple fusion receptors with three signalling motifs have been produced [24,32]. Once the efficacy of compound signalling motifs was understood, new third-generation CAR were developed utilizing multiple pathways. For example, the introduction of 4-1BB (CD137) domains enhanced CAR-transduced T cell survival and function by inducing production of the anti-apoptotic protein Bcl-x [33]. The order in which the signalling moieties are placed in sequence has also been shown to have an impact on CAR function. Recent studies indicate that CAR using the CD3ζ transmembrane domain are incorporated into the host T cell TCR/CD3 complex which is important for optimal function of the CD3ζ CAR [29]. However, the CD28 co-stimulatory domain does not function effectively when placed downstream of the CD3ζ transmembrane domain [30,34]. Thus, these findings indicate that all aspects of the CAR require more detailed examination. Multiple formats of CAR signalling domain have been published, each showing potential advantages over competitors *in vitro* and *in vivo*. However, as yet no single CAR has been accepted universally as being the 'optimal' configuration, with most research groups (somewhat understandably) using their own preferred CAR. The major issue is that for clinical application there is no standard accepted CAR configuration, making it difficult to compare the results effectively from the wide number of currently ongoing clinical trials.

None the less, recent exciting clinical studies have indicated the potential of CAR T cells. Three patients with chronic lymphocytic leukaemia treated with CD19/4-1BB/CD3ζ CAR T cells had strong anti-tumour responses and encouraging *in vivo* expansion of the CAR T cells [35]. In another trial a single patient with follicular lymphoma demonstrated tumour regression after treatment with CD19/CD28/CD3ζ CAR T cells with a long (39-week) persistence of CAR T cells post-adoptive transfer [36]. Results from a third, much larger trial have recently been published outlining the safety and persistence of second-generation CAR in patients with relapsed B cell leukaemias, and demonstrated clear trafficking of CAR T cells to tumour sites [37].

While there are several trials currently under way in centres around the world targeting B cell malignancies through the CD19 receptor, the clinical investigation of CAR targeting non-B cell tumours is less advanced. T cells engrafted with a first-generation CAR specific for the alpha folate receptor on ovarian tumours cells failed to persist in five patients and showed no positive clinical effect [38]. Several other studies targeting antigens expressed by solid tumours such as CEA are under way. However, a single patient treated with a Her2/neu-specific CAR with a CD28–CD3ζ signalling domain died as a result of on-target toxicity, resulting in a cytokine storm and subsequent organ failure [39]. On-target toxicity was also observed in a European trial

employing a CAR specific for carbonic anhydrase IX with the suggestion that bile duct cells were targeted by the CAR, leading to symptoms of jaundice and altered liver function [40]. In this case, systemic steroid treatment resolved the symptoms, due probably to eradication of the gene-modified T cells.

The utilization of these CAR-based T cell therapies in clinical trial provides some encouragement in terms of early observations of clinical responses. However, as with TCR-based T cell therapy, there are some warnings of the potency of the CAR T cell with on-target toxicities being observed in certain situations. With the development of more potent signalling domains to drive CAR T cell function, ensuring that toxicity is controlled will be a major clinical objective for the foreseeable future.

Bi-functional T cells

Both TCR and CAR have been transferred to naive T cells to drive targeting of TAA-specific cells, producing tumour-specific redirected T cells. More recently populations of T cells with predefined antigen specificity have been considered for use. TAA-specific TCRs or CAR can be transferred into T cells already targeted against common and persistent viruses such as cytomegalovirus (CMV) or Epstein–Barr virus (EBV) to create bi-functional T cells with specificities for both virus and tumour (Fig. 2) [41,42]. It is believed that the endogenous TCR will recognize viral antigens frequently *in vivo* resulting in proliferation and cytotoxic activity via both receptors which will help to maintain both function and persistence [26]. It has been shown that it is far easier to reach the threshold required for effective T cell responses through the endogenous TCR than it is for the introduced TCR, which highlights the advantage of bi-functional T cells compared to other engineered T cells [26].

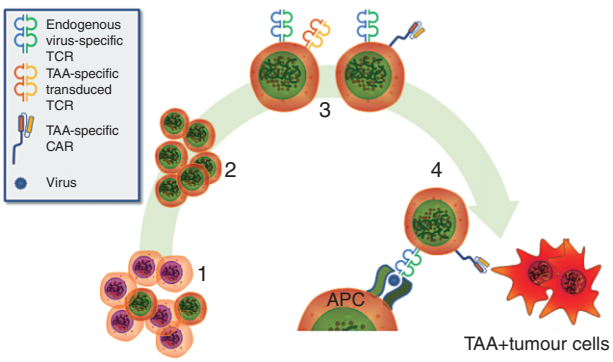

Fig. 2. Bi-functional T cells can be generated by (1) isolating donor virus-specific T cells then (2) expanding them *in vitro* [41]. These T cells can then be (3) transduced either with tumour-specific T cell receptors (TCRs) or chimeric antigen receptor (CAR), expanded and returned to the donor [41]. The endogenous TCR recognition of virus via antigen-presenting cells (APC) maintains survival and (4) enhances anti-tumour activity of the transduced T cells [41,42].

Although virus-specific T cells may be relatively rare in immunocompetent donors, efforts have focused on methods for selecting these cells directly from donor blood [43]. It is possible that CTLs specific for multiple latent viruses could also be isolated from a single patient, increasing the cost-effectiveness of this procedure [43,44]. The diverse TCR repertoire present in peripheral blood mononuclear cells (PBMC) increases the likelihood of TCR dimers with unknown specificity being formed; there is also a possibility of transduction into regulatory T cells (T_{regs}), potentially inhibiting the tumour-specific immune response [42]. This can be overcome by not only excluding T_{regs} but also selecting for latent virus-specific CD8 T cells which have maximal TCR expression and restricted TCR usage [42,45].

Bi-functional T cells are capable of maintaining an anti-tumour response to a TAA even when the antigen is down-regulated in the tumour environment [46]; the support from virus antigen-presenting cells sustains the proliferation, survival and function of the bi-functional cells through the endogenous TCR [12,45,47,48]. Surface expression of transduced TCR or CAR can reduce over time, but in bi-functional T cells exposure to latent viruses maintains the strong, sustained T cell response. This can be driven by the fact that, even in asymptomatic infected individuals, up to 2% of T cells can be virus-specific [12,48].

The first successful demonstration of bi-functional T cells being used to mount an immune response against a TAA was shown by Murphy *et al.* in 2007 using T cells specific for both influenza and the melanoma TAA Erbb2 [47]. In 2008 Pule *et al.* reported that bi-functional T cells offered improved therapeutic potential by demonstrating that EBV-specific T cells transduced with a first-generation GD2 CAR (a glycolipid TAA associated with neuroblastoma) persisted longer *in vivo* than poly-specific T cells transduced with the same CAR. These bi-functional T cells also mediated tumour necrosis or regression (one complete remission) and on re-isolation *ex vivo* were able to be re-expanded *in vitro* with EBV-specific B cell lines for up to 6 months after transfer [49]. The antigen GD2 is also expressed on central nervous system (CNS) tissues, including neurones, and the fact that toxicity was not observed in this study suggests that bi-functional T cells may respond to tumour and normal cells differently [48,49]. Also noted was the lack of bi-functional T cells in areas of tumour regression, suggesting that these cells may function mainly at the start of an immune response to recruit other cells to the area which themselves facilitate tumour regression [48,49].

The use of bi-functional T cells as tumour therapy confers additional protection benefits. During immunosuppression following patient conditioning, latent viruses may recrudesce, causing severe disease or death. Bi-functional T cells 'kill two birds with one stone' as constant stimulation of the endogenous virus-specific TCR mediates an immune

response to both virus and tumour [29]. This has been demonstrated in varicella zoster virus (VZV)-specific transduced cells which recognize and eradicate tumour cells while preventing VZV reactivation in immunosuppressed patients [50].

Important considerations for generating redirected-specificity T cells

Antigen choice

In the optimal situation, the target antigen is tumour-specific with no significant expression on normal tissues. Fusion proteins (such as bcr-abl) and altered tumour-specific proteins [such as mucin 1 (MUC-1)] may be examples of such targets, and indeed MUC-1 has been targeted with both CAR and TCR transfer [13,28]. However, these target antigens are relatively rare, and in most cases may be patient-specific, ruling out the possibility of using generic targeting receptors for therapy. Most of the current defined tumour antigens are TAA which have reduced or organ-specific expression in normal healthy tissue. Targeting of this healthy tissue has already raised on-target toxicity issues, as described from TCR and CAR clinical trials above. Understanding the rules that control targeted T cell toxicity to the tumour while sparing normal tissue is a central issue for the development of this approach. However, several further issues relate to the choice of target. For the CAR approach, the target antigen must be expressed on the cell surface so that it can be accessed by the scFv moiety. It is no accident that B cell lymphoma has been the target of choice for a number of current CAR T cell trials. B cells reside in the same anatomical location as T cells and express a number of receptors that facilitate T cell function such as co-stimulatory receptor ligands. In order to access antigens associated with solid cancers, gene-modified T cells need to traffic to the tumour site and overcome both physical barriers and a highly immunosuppressive micro-environment. Lack of co-stimulatory ligands on, for example, epithelial tumour target cells means that it will not provide the required signals to mediate effective T cell function, while the heterogeneity of target antigen expression on the tumour could mean incomplete T cell activation and reduced therapeutic activity. This heterogeneity of TAA expression is also problematic for dual-specificity T cells [51]. In the face of these issues, it is easy to imagine that more potent receptors to drive gene-modified T cell function would be required in the harsh setting of the solid tumour. However, this raises the possibility of unwanted auto-toxicity. The development of more sophisticated animal models that can permit the exploration of these issues is becoming of increasing importance.

T cell type

Although the chosen tumour antigen is vital to the effectiveness of the redirected-specificity therapy, the role of the T cell carrying the effector receptors cannot be underestimated. Significant study has gone into assessing which T cell subset represents the most effective for the generation of redirected-specificity T cells.

The majority of the published redirected or bi-functional T cell studies utilize CD8 T cells as CD8 populations are reduced significantly in many cancer patients. These introduced T cells can induce patient CD8 T cells to switch to CTLs of the same specificity as the transduced TCR [52]. This also requires transduction of CD4 T cells, as they provide help in the form of co-stimulation and are required for effective CD8 responses and memory formation [53].

Less differentiated T cells are preferred for use in adoptive transfer as both T cell survival and tumour regression correlates with the longer telomeres found in early differentiation T cells [54,55]. Another rationale for using naive cells is shown by the expression of KLRG1 (a marker of lower proliferative potential due to defective Akt phosphorylation), which is expressed in effector memory T cells *in vitro* and *in vivo* but not in naive T cells [56]. However, a recent study assessing bi-specific T cell function in three different T cell populations (EBV-specific CTL, cytokine-induced killer T cells and gamma–delta T cells) showed no difference in functional capability [57]. Therefore, it suggests that further research is required to identify the optimal cell population for generating dual-specificity T cells.

Vector

The effectiveness of the transfected T cell relies on the efficiency with which the target T cells can be transduced with the TAA-specific TCR or CAR, and this depends entirely on the vector. The ideal transduction vector should have high efficiency resulting in stable expression of the introduced TCR or CAR; it is also preferable to have a low number of integrations to prevent inappropriate gene insertion and potential mutagenesis in the host genome [19]. Ideally, the vector would permanently integrate the TCR/CAR into the cells, although methods investigating the use of transient CAR expression through mRNA transfer have been proposed [58]. The different vector options are outlined in Table 2, with a number of these methods still undergoing clinical trials.

Conditioning

Once the chosen T cells have been transduced successfully, they need to be expanded and then transfused back into the recipient. The transfer is usually preceded by intensive chemotherapy known as conditioning. This functions to 'make space' for the newly administered cells by reducing the number of host lymphocytes (including T_{regs}) which compete with the newly transferred cells for homeostatic cytokines [23]. The transfused redirected or bi-functional T cell numbers should increase rapidly in the patient through homeostatic expansion via IL-7 and IL-15 [23], although this

Table 2. Vector options for generating bi-functional T cells.

Vector	Method	Advantages	Disadvantages
Transposon	Non-viral	Transposons such as the Sleeping Beauty (SB) system can introduce CAR/TCR genes into genome SB-transduced cells show high gene expression, specific cytotoxicity, production of Th1 cytokines, and mediate tumour reduction [75,76]	Triggering (*in vitro* stimulation using anti-CD3/CD28) is required prior to transduction which may affect cell efficacy [77]
		Simple and inexpensive, and does not require cell preactivation, reducing culture time [73,75]	Not yet tested clinically [76]
Lentiviral vectors	Viral	Self-inactivating, with lower risk of damaging insertions and can carry a large payload [19] Permanently integrates into host genome and capable of integrating into non-dividing cells [52] Efficient, lowered culture time, requires only cytokines (IL-15) post-transduction [78] Transduced T cells are relatively undifferentiated, which may lead to improved function *in vivo* [78]	Triggering may still be required to produce significant levels of modified cells in some cases [19]
Retroviruses (generally for TCR transfer)	Viral	Permanent gene expression due to integration into host genome [52] Self-inactivating vectors have been constructed and may be useful for therapy [19] γ-Retroviral vectors require triggering and expansion with IL-2 [78]	Tend to insert near transcription start sites, potentially activating proto-oncogenes [52]
Recombinant adenovirus	Viral	Can integrate into both dividing and non-dividing cells Some successes with TCR transfer, and continue to be used in clinical trials Selective integration is possible [52]	Require receptor-mediated entry into cell. However, T cells express low viral receptor levels – integration may be blunted [52]

CAR, chimeric antigen receptor; IL, interleukin; TCR, T cell receptor; Th1, T helper type 1.

expansion may be compromised by high tumour burdens [37]. The cytotoxic conditioning regimen has a dual function, in that it may also kill a proportion of the tumour cells, releasing antigen and enhancing tumour recognition [59]. There is also evidence that irradiation enhances adhesion molecule expression, facilitating movement of the TAA-targeted T cells into tumour sites [59,60]. However, the conditioning regimen may also pose a risk of morbidity or mortality to the patient, so selective depletion of lymphocytes post-transfer may offer a safer option [23]. Other options involve enhancing the responsiveness of the redirected or bi-functional T cells to cytokines such as IL-7 or IL-4, thereby promoting *in vivo* survival and expansion [61,62].

Adapting redirected T cells to conditions encountered *in vivo*

A key issue for the use of redirected T cells is the maintenance of persistence and function against the target cancer. Tumours harness several protective mechanisms to minimize immune attack and subvert immune responses, and supplemental methods have been investigated to enhance the function of the introduced T cells.

Transfusion of redirected-specificity T cells is often accompanied by administration of growth factors [such as granulocyte–macrophage colony-stimulating factor (GM-CSF)] and Toll-like receptor (TLR) agonists [such as poly(I:C) or cytosine–guanine dinucleotide (CpG)] to enhance immune stimulation and anti-tumour responses [63]. Although interleukins such as IL-2 may be administered following transfer to promote T cell proliferation, it has severe side effects and conversely may also hamper effective immune responses by expanding forkhead box protein 3 ($FoxP3^+$) T_{regs} [61,63]. There is some evidence that IL-15 (which expands CD4 and CD8 T cells) might be useful, as it has similar properties to IL-2, but is less toxic and does not induce T_{reg} proliferation [64]. However, IL-15 administration may result in profound neutropenia and its use in an already immunosuppressed patient would need to be considered carefully [64].

A key issue with the use of tumour-specific adoptive T cell therapy is how long the cells remain within the body. Persistence may be enhanced by introducing TAA-specific TCR-αβ genes into haematopoietic stem cells, which would result in continuous production of the enhanced T cells through homeostasis [59]. Although there has been some success with this method, it is unclear whether the transduced cells would persist following thymic education [59]. Another issue is the potential for malignant transformation [65,66].

Introducing tumour-specificity into γδ T cells represents an intriguing possibility, as they have a known role in

tumour immunosurveillance, are cytotoxic to many tumour types and can effectively prime αβ T cells. The use of γδ T cells also eliminates the risk of chain mispairing, as γδ chains would be unable to pair with the introduced TAA-specific αβ chains [67]. Evidence has shown that γδ T cells show clear anti-tumour effects after transduction [57,67].

One issue that redirected or bi-specific T cells will face in the patient is a profoundly immunosuppressive microenvironment around the tumour. Much of this is mediated through release of TGF-β, which can attenuate T cell function. It has been shown recently that EBV-specific CTLs can be transduced with a retroviral vector carrying a dominant negative TGF-β type II receptor (DNRII) which functions to block the immunosuppressive effects of TGF-β while retaining the killing ability of the transduced cell, resulting in significant tumour regression [68]. However, TGF-β is pleiotropic in function and is required for other aspects of immune regulation, and although there are concerns that the introduction of a DNRII would result in uncontrolled immune responses, this has not been observed [68].

Another target receptor is the type I IL-13 receptor (IL-13Rα2), which is overexpressed on the surface of a number of solid tumours and tumour cell lines *in vitro*, including ovarian and renal cell carcinoma. The receptor is generally present at very low levels on normal tissues. In a rodent model, vaccination with IL-13Rα2 DNA delayed tumour growth due to a CTL response against IL-13Rα2, and correlates have been seen in human tumours [69]. Therefore blocking or targeting IL-13Rα2 may provide further support to therapy using redirected or bi-specific T cells [69].

Conclusion

Cancer is a major threat to health in the 21st century, and the intense interest in harnessing immune mechanisms to control disease has led to the development of promising novel T cell therapies for treatment. These utilize the potent cytotoxic and cytostatic functions of T cells, but improve tumour targeting by transducing in receptors specific for tumour antigens. One of the main limitations on the use of redirected-specificity T cell therapy is the extensive guanosine monophosphate (GMP)-level *in vitro* culture required for transduction and expansion, which often results in terminally differentiated cells with poor survival and function *in vivo* [70]. There is also evidence that in culture non-transduced cells may impact upon the function of the transduced cells [12]. At present, the cost of redirected or bi-functional T cell therapy is estimated to be approximately $100 000 per patient, although it is thought that this could be quite easily reduced to around $20 000, which would make it competitive with other therapies [63]. The effectiveness of redirected T cells as a cancer immunotherapy is evident from the substantial tumour regression and even eradication seen in animal models and preliminary clinical trials. In particular, the use of bi-functional T cells allows the immune response against a latent virus to be harnessed and redirected against a defined TAA leading to a strong, sustained immune response to the tumour. With the continuing clinical trials and introduction of next-generation constructs, research will hopefully lead to effective treatments. Addressing the confounding issues discussed in this review should result in improvements in efficacy to such a level that redirected or bi-functional T cell therapies will become fully transferred from the laboratory to the patient.

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